



# COMPARISON OF DIFFERENT PHENOTYPIC METHODS FOR THE DETECTION OF EXTENDED SPECTRUM $\beta$ - LACTAMASE (ESBL) IN BACTERIAL ISOLATES FROM TERTIARY CARE CENTRE

Shaikh N. K.<sup>1</sup>, Mundhada S. G.<sup>2</sup>, Lalngaihuali R.<sup>3</sup>, Ingole K. V.<sup>4</sup>

<sup>1</sup>Associate Professor, Department of Microbiology, Dr. V. M. Government Medical College, Solapur -413003, Maharashtra, India; <sup>2</sup>Assistant Professor, Department of Microbiology, Dr. V. M. Government Medical College, Solapur -413003, Maharashtra, India; <sup>3</sup>Junior Resident, Department of Microbiology, Dr. V. M. Government Medical College, Solapur -413003, Maharashtra, India; <sup>4</sup>Professor & Head, Associate Professor, Department of Microbiology, Dr. V. M. Government Medical College, Solapur -413003, Maharashtra, India.

## ABSTRACT

**Background and objective:** Extended spectrum  $\beta$  lactamases (ESBLs) continue to be a major problem in clinical setups. Their detection is essential for proper antibiotic therapy, to limit the spread of resistance mechanisms and for epidemiological purposes. The objective of our study was to compare different phenotypic methods for detection of ESBL to know the best suitable one in our setup.

**Material and method:** A total of 127 Gram negative isolates were identified by standard protocol and Antibiotic susceptibility testing (AST) was done. Isolates which were resistant to one of the third generation cephalosporins were selected provisionally as ESBL producers and then subjected for confirmation by Phenotypic confirmatory disc diffusion test (PCDDT), Double disk synergy tests, Modified double disk synergy test (MDDST), Indirect modified three dimensional test (IMTDT) to evaluate their ability to detect ESBLs. Minimal inhibitory concentration (MIC) by the agar dilution method was used as the standard reference method.

**Result:** MIC detection by agar dilution method had confirmed all 63(49.60%) screening positive isolates as ESBL producers. E.coli which constitute 39(61.90%) of the isolates was found to be highest ESBL producer among them. PCDDT by CLSI detected ESBL in 55(87.30%) isolates. DDST using amoxicillin-clavulanic acid detected the same in 49 (77.78%) cases. MDDST using cefepime & piperacillin-tazobactam detected ESBL in 60(95.23%) cases. IMTDT detected 62(98.41%) ESBL producing isolates.

**Conclusion:** IMTDT was found to be superior method than MDDST, PCDDT and DDST for detection of production of ESBL alone or in presence of other  $\beta$ - lactamases like Amp C.

**Key Words:** Amp C, DDST, ESBL, IMTDT, MDDST, PCDDT

## INTRODUCTION

The most common cause of bacterial resistance to  $\beta$ -lactam antibiotics is the production of enzyme  $\beta$ -lactamases. Over the last 20 years, many new  $\beta$ -lactam antibiotics have been developed which were specifically designed to resist to the hydrolytic action of  $\beta$ -lactamases. However, with each new class that has been used to treat patients, new  $\beta$ -lactamases emerged that caused resistance to that class of drug.<sup>1</sup>The latest in the series of these enzymes is the evolution of Extended-

ed Spectrum Beta Lactamases (ESBLs) due to extensive use of 3<sup>rd</sup> generation cephalosporins. These ESBLs efficiently hydrolyze oxyimino cephalosporins conferring resistance to third generation cephalosporins and to monobactams.<sup>2</sup> Plasmids coding for ESBLs may also carry additional  $\beta$ -lactamase genes as well as genes conferring resistance to other antimicrobial such as quinolones, aminoglycosides and sulphonamides. This can limit the chemotherapeutic options for ESBL-producing pathogens and facilitate the inter and intraspecies dissemination of ESBLs.<sup>3</sup>

### Corresponding Author:

Mundhada S. G., Department of Microbiology, Dr. V. M. Government Medical College, Solapur -413003, Maharashtra, India.

E-mail: sapana.mundhada@gmail.com

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Considering the increasing rate of infections caused by ESBL producing organism, their detection is essential for prescribing appropriate antibiotic therapy, to limit the spread of resistance mechanisms and for epidemiological purposes. Various methods like phenotypic (disk diffusion, disk potentiation, double disk synergy procedure, three dimensional test and E-test ESBL strip), semi-automated [Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD), the VITEK 2 System (bioMérieux, Marcy l'Etoile, France), and the MicroScan Walk Away-96 System (Dade Behring, Inc., West Sacramento, CA)] and molecular methods (PCR, PCR-RFLP and nucleotide sequencing) have been described for ESBL detection; however, each method has its own limitations.<sup>4</sup>In the present study, we compared different phenotypic methods for detection of ESBL to know the best suitable one in our setup.

## MATERIAL AND METHOD

The present study was carried out at a tertiary care centre in the Microbiology department from September 2015 to February 2016. Total 127 gram negative bacteria were isolated from various samples including blood, pus, surgical site wounds, burn wounds, tracheal aspirates, central venous pressure tips and urine. Identification of isolates was done based on cultural characteristics and reaction in standard biochemical tests.<sup>5,6</sup> Antimicrobial sensitivity testing was done by the Kirby-Bauer disc diffusion method.<sup>7</sup>ESBL detection was done in those Gram negative isolates which revealed resistance/decrease susceptibility to 3rd generation Cephalosporin i.e. Ceftazidime (30 $\mu$ g) or Cefotaxime (30 $\mu$ g) or both according to CLSI guideline (Screening test).Detection of minimum inhibitory concentration (MIC) by the agar dilution technique for 3<sup>rd</sup> generation cephalosporins with and without clavulanic acid in accordance to CLSI guidelines<sup>7</sup> was taken as standard reference method for comparison purpose with different phenotypic method for detection of ESBL as given below.

**1. Phenotypic confirmatory disc diffusion test (PCDDT)<sup>7</sup>**– It is performed according to CLSI guideline. Ceftazidime (30  $\mu$ g) discs alone and in combination with clavulanic acid (ceftazidime + clavulanic acid, 30/10  $\mu$ g discs) were placed on a Muller Hinton agar (MHA) plate which was inoculated with the test strain. An increase of  $\geq$  5mm in the zone of inhibition of the combination discs in comparison to the ceftazidime disc alone was considered to be an ESBL producer.

**2. Double disk synergy test (disk approximation test)<sup>8</sup>**– MHA plate was inoculated with test strain. Augmentin (20 $\mu$ g amoxicillin + 10 $\mu$ g clavulanic acid) disc was placed in centre of plate. Disc of cefpodoxime (30 $\mu$ g), ceftazidime (30 $\mu$ g) and cefotaxime (30 $\mu$ g) were placed 15-20 mm (centre to centre) around augmentin disc. Enhancement in zone of in-

hibition of any cephalosporin toward augmentin indicated presence of ESBL producing organism.

**3. Modified double disk synergy test (MDDST)<sup>8</sup>**– The original double disk synergy test was modified for detecting ESBL in AmpC producing clinical isolates. A disc of augmentin was placed in the centre of MHA; then discs of cefpodoxime (30 $\mu$ g), ceftazidime (30 $\mu$ g), cefotaxime (30 $\mu$ g), aztreonam (30 $\mu$ g) and cefepime (30 $\mu$ g) were kept 16 to 20 mm (centre to centre) around augmentin disc. From the cefepime disc at a distance of 22 to 25 mm centre to centre disc of piperacillin-tazobactam (100/10 $\mu$ g) was placed. The organisms were considered to be ESBL producer when the zone of inhibition around cefepime or any of the extended-spectrum cephalosporin discs showed a clear-cut increase towards the piperacillin-tazobactam (PIT) disc or augmentin disc.

**4. Indirect modified three dimensional test (IMTDT)<sup>9</sup>**– MHA plate was inoculated with standard sensitive strain (*E. coli* ATCC 25922) matching 0.5 McFarland turbidity standards. A disc of 3<sup>rd</sup> generation cephalosporin (ceftazidime/ceftriaxone/cefotaxime) or aztreonam was placed in the centre of the plate. At a distance of 2 mm from the antibiotic disc a well of 4 mm (diameter) was punched and inoculum (30 $\mu$ L) of the test strain preadjusted to 5.0 McFarland standards was seeded into the well. Heart shaped distortion of zone of inhibition around the  $\beta$ -lactam disc was indicative of an ESBL production.

**Quality control** –*Klebsiella pneumonia* ATCC 700603 and *Escherichia coli* ATCC 25922 were used as ESBL positive and negative controls, respectively.

## RESULT

Among the 127 gram negative bacteria isolated, 63(49.60%) were resistant or revealed decreased susceptibility to 3<sup>rd</sup> generation cephalosporin on screening giving provisional diagnosis of ESBL. All screening positive 63 isolates were confirmed as ESBL producers by standard reference method. (MIC detection by the agar dilution technique) ESBL production was seen highest in *E. coli* isolates 39(61.90%) followed by *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Proteus species*, *Citrobacter species* and *Acinetobacter species*. (Table I) Antibiotic Resistance pattern of ESBL producing isolates is as shown in Table II. Imipenem, Piperacillin-Tazobactam and Amikacin were found to be most sensitive antibiotic while maximum resistance was observed against cefotaxime, ceftazidime, Trimethoprim-sulfamethoxazole and Tetracycline. Prevalence of ESBL production in different isolates in present study was detected by various phenotypic methods. Table III shows evaluation of these phenotypic methods in comparison to reference standard method (MIC detection by Agar dilution).

## DISCUSSION

The prevalence of ESBL among gram negative bacteria constitutes a serious threat to current  $\beta$  lactam therapy leading to treatment failure and consequent escalation of costs of hospital stay. Many clinical laboratories are not fully aware of the importance of the ESBL producers and of methods to detect them. This lack of understanding is responsible for a continuing failure to respond appropriately to prevent the rapid, worldwide dissemination of the pathogens which possess these  $\beta$ -lactamases. Hence, ESBL detection must be efficient to aid in formulating an antibiotic policy and containment measures to solve the issue of antibiotic resistance.<sup>10</sup>

Previous studies from India have reported the prevalence of the ESBL producers to be 6.6 to 68%.<sup>11, 12, 13, 14</sup> In the present study 49.60 % of gram negative bacteria were ESBL producers. *E.coli* was the predominant ESBL producer in our study. Similar finding were reported by Kulkarni et al and Ananthakrishnan et al.<sup>15, 16</sup> We observed that a majority of the isolates in our study were susceptible to imipenem (100%), Amikacin (77.77%) and piperacillin-tazobactam (73.02%). Studies by Dalela et al<sup>17</sup> and Baby et al<sup>18</sup> also observed similar result. We observed that imipenem is the most active drug for the treatment of infections caused by ESBL producers, followed by Amikacin and piperacillin/tazobactam. These drug should be used as reserved one, their indiscriminate use should be minimize to prevent emergence of resistance.

Different methods were used in present study for detection of ESBL production. PCDDT, the confirmatory method recommended by CLSI had detected ESBL production in 87.30% of isolates. While DDST, using Amoxiclav as inhibitor of ESBL showed positive result in 77.78% of isolates. In present study between these two tests, PCDDT was more sensitive than DDST. Similar finding were observed in other studies by Khan et al and Modi et al.<sup>8, 9</sup> Sensitivity of both PCDDT and DDST were less compared to standard reference test because Clavulanic acid which was used as ESBL inhibitor in PCDDT & DDST act as inducers of high level AmpC production and it led to resistance to 3rd generation cephalosporins as well 3rd generation cephalosporins + clavulanic acid. So even if ESBL was present, it would not be detected and resulted in false negative test.<sup>9</sup>

For MDDST, few modifications were done in DDST like addition of piperacillin- tazobactam as ESBL inhibitor, use of 4th generation cephalosporins (Cefepime) & optimum spacing of drugs for detection of synergy. If AmpC was present, cefepime would be sensitive so synergy could be seen with 4th generation cephalosporins & inhibitor antibiotic.<sup>8</sup> Tazobactam & sulbactam were less likely to induce AmpC B-lactamase compared to clavulanic acid. So, simultaneous Amp C induction did not occur. In present

study, MDDST had detected 60(95.23%) isolates which is 11 isolates more than detected by DDST. MDDST found to be more sensitive than DDST and PCDDT in our study. These finding can be correlated with observation made by Modi et al<sup>9</sup> in their study.

In present study, IMTDT identified 62(98.41%) ESBL positive isolates. Thus it was the most sensitive test among all other tests. It was superior in detecting ESBL than PCDDT, DDST and MDDST in our study. Other studies carried out by Menon et al<sup>19</sup> and Modi et al<sup>9</sup> also reported similar observations. Thus, PCDDT & DDST should be used in the isolates which produce only ESBL but are not useful for detection of ESBL in isolates who also produces other  $\beta$ -lactamases like AmpC enzyme. While MDDST due to modification in it and IMTDT had showed good sensitivity when compared to standard reference method.

## CONCLUSION

Infections caused by ESBL producers often limit therapeutic options and cause treatment failures. Thus, detection of ESBL production should be performed routinely in microbiology laboratories so that the appropriate antimicrobial therapy can be instituted and the dissemination of ESBL producers may be prevented by employing appropriate infection control measures. It is important to increase awareness of among physician about ESBL producers and different methods available to detect them. Molecular assays may provide accurate results in the identification of ESBL genes, but their accessibility is often limited and they are expensive. Phenotypic methods are easy to perform and interpret. In our study, IMTDT was found to be superior method than MDDST, PCDDT and DDST for detection of production of ESBL alone or in presence of other  $\beta$ - lactamases like AmpC.

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**Table I: Distribution of Isolates among ESBL producer (n=63)**

Organism	Number (%)
E.coli	39(61.90)
Klebsiella pneumonia	13(20.63)
Paeruginosa	6(9.52)
Proteus species	3(4.76)
Citrobacter species	1(1.58)
Acinetobacter species	1(1.58)
Total	63

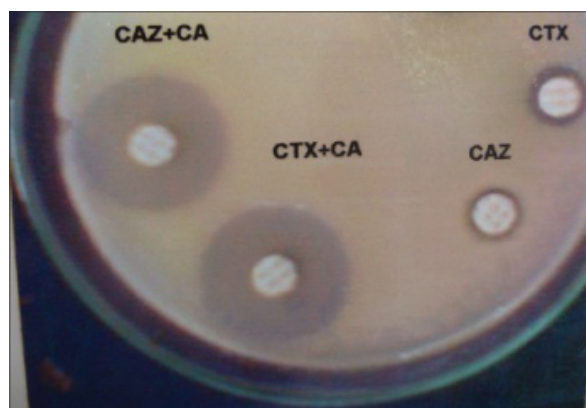
**Table II: Antibiotic Resistance pattern of ESBL producing isolates (n=63)**

Antibiotic	Resistance (%)
Cefotaxime	100
Ceftazidime	100
Tetracycline Amikacin	87.30
Trimethoprim- sulfamethoxazole	84.13
Ciprofloxacin	65.07
Pipracillin-Tazobactam	26.98
Amikacin	22.23
Imipenem	0

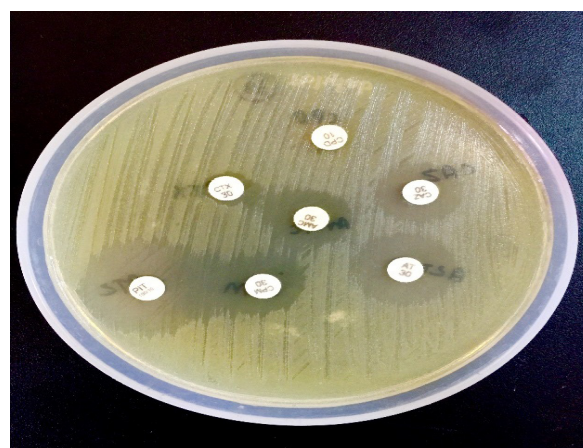


**Table III: Result obtained by Comparison of different ESBL detecting phenotypic methods (n=63)**

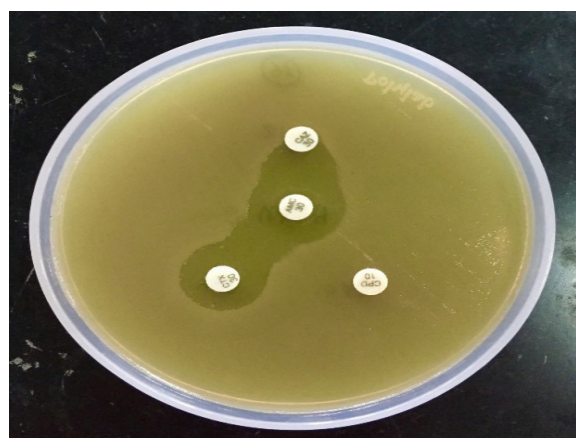
Phenotypic methods	ESBL producers (%)
MIC by agar diffusion (standard reference method)	63 (100%)
Phenotypic confirmatory disc diffusion test (PCDDT)	55(87.30%)
Double disk synergy test(DDST)	49(77.78%)
Modified Double disk synergy test(MDDST)	60(95.23%)
Indirect modified three dimensional test (IMTDT)	62(98.41%)



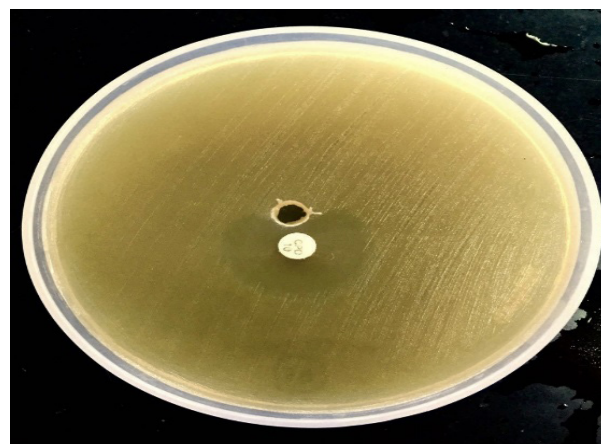
**Photograph 1:** Positive Phenotypic confirmatory disc diffusion test by CLSI for ESBL detection.



**Photograph 3:** Positive modified double disc synergy test for ESBL detection. [Showing synergy between cefepime (CPM) and Pipracillin-tazobactam (PIT). Amoxicillin-clavulanic acid showing synergy only with cefotaxime (CTX)].



**Photograph 2:** Positive Double disc synergy test for ESBL detection. [Showing synergy between amoxicillin-clavulanic acid(AMC) and indicator antibiotic like ceftazidime(CAZ) and cefotaxime(CTX)].



**Photograph 4:** Positive modified indirect three dimensional test for ESBL [Showing heart shape distortion around ceftazidime disc near well inoculated with suspension of test organism].